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TITLE: Epigenetic Mediation of Endocrine and Immune Response in an Animal Model of Gulf War Illness

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14. ABSTRACT There are now compelling human epidemiological and animal experimental data that indicate the risk of developing complex diseases is influenced by persistent epigenetic adaptations in response to environmental exposures such as toxins and stress. We propose to examine the epigenomic response to diisopropyl fluorophosphates (DFP), a sarin surrogate, and associated changes to the immune and endocrine response to lipopolysaccharide (LPS) challenge in a mouse model of Gulf War Illness (GWI), with stress hormone exposure as an experimental mediator. We will study the relationship between changes in DNA methylation and chromatin modifications in peripheral blood and the brain (specifically hippocampus and prefrontal cortex) in order to pursue a mechanistic understanding of the underlying pathology of GWI. During this reporting period, we have begun data collection on DNA methylation modifications and gene expression profiles in the brains of mice exposed to saline control, corticosterone and DFP, and have developed and refined protocols for this project in line with the GWIRC sister project.					
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Introduction

Epigenetic changes, including modifications to chromatin structure and DNA methylation, alter gene expression and cellular phenotype in the absence of variation in DNA sequence. There are now compelling human epidemiological and animal experimental data that indicate the risk of developing complex diseases is influenced by persistent epigenetic adaptations in response to environmental exposures such as toxins and stress. We propose to examine the epigenomic response to diisopropyl fluorophosphates (DFP), a sarin surrogate, and associated changes to the immune and endocrine response to lipopolysaccharide (LPS) challenge in a mouse model of Gulf War Illness (GWI), with stress hormone exposure as an experimental mediator. We will study the relationship between changes in DNA methylation and chromatin modifications in peripheral blood and the brain (specifically hippocampus and prefrontal cortex) in order to pursue a mechanistic understanding of the underlying pathology of GWI. Epigenetic profiles indicative of transcriptional enhancement or repression may help identify stable biomarkers of GWI related to exposure across a variety of environmental conditions. Because epigenetic marks are potentially reversible, elucidating the manner in which environmental interventions including immune-endocrine stressors alter epigenetic marks across the blood-brain barrier in the context of toxin exposure will offer insight into mechanisms leading to stable disease states and novel routes to therapeutic intervention. The overall objective of this project is to identify epigenetic mechanisms of altered Hypothalamic-Pituitary-Adrenal (HPA) axis and immune signaling in a mouse model of environmental exposures linked to GWI. DNA methylation and histone modifications will be examined in peripheral blood and the brain using a high-throughput genome-wide approach. This proposal adds substantial value to a funded GWIRP Consortium project designed to examine gene regulatory dynamics in a model of toxicant exposure (DFP, a sarin surrogate) and stress/immune challenge (CORT, LPS).

Keywords

Gulf War Illness, Hypothalamic-Pituitary-Adrenal (HPA) axis, epigenetic, hippocampus, prefrontal cortex, lipopolysaccharide (LPS), diisopropyl fluorophosphate (DFP), corticosterone (CORT), peripheral blood mononuclear cell (PBMC), DNA methylation, Histone acetylation, immune system, genomics, mouse model

Accomplishments

What were the major goals of the project?

- 1) The specific objectives described in the approved SOW were:

Specific Aim 1: Assess epigenetic changes in peripheral blood in response to DFP exposure concomitant with sustained corticosterone stress hormone administration and subsequent antigen (LPS) challenge

Specific Aim 2: Assess epigenetic changes in brain as a function of DFP exposure, corticosterone administration and in response to LPS challenge

Specific Aim 3: Assess the relationship between epigenetic changes and gene regulatory dynamics in brain and in blood, and their relationship to transcriptomic and physiological outcomes

2) The project tasks and associated as stated in the approved Statement of Work (SOW) were:

Task 1: Validate mouse model and perform manipulations of animal subjects.

- Milestone: IACUC/ACURO Approval.

Task 2: Extraction of tissue samples and blood sample processing.

- Milestone: Validation of manipulation and collection of tissue samples
- Milestone: DNA from blood prepared for Sequencing
- Milestone: DNA library preparation for sequencing of blood
- Milestone: Blood epigenetic paper

Task 3: Extraction of tissue samples and brain sample processing.

- Milestone: DNA from brain prepared for Sequencing
- Milestone: DNA library preparation for sequencing of brain
- Milestone: Brain epigenetic paper

Task 4: Gene expression analysis on blood and brain

- Milestone: Gene expression profiles for blood, hippocampus and prefrontal cortex

Task 5: Analysis of epigenetics and gene expression across tissue types

- Milestone: Identify epigenetic correspondence across tissue types
- Milestone: Functional epigenetics paper

What was accomplished under these goals?

Major Activities and results during this reporting period:

Task 1. Validate mouse model and perform manipulations of animal subjects.

Subtask 1. Obtain regulatory approval.

Our IACUC renewal for this reporting period was submitted June 1 2016 and was approved Oct 21 2016 by Co-PI Dr. O’Callaghan of the CDC. The renewal of our ACURO is currently being prepared for submission by Dr. O’Callaghan.

Subtask 2: Perform baseline and challenge studies on animal models.

Animal dosing: We have obtained tissue samples for comparison conditions T0, T1, and T2. Tissue samples, consisting of blood peripheral mononucleotide cells (PBMCs) and brain from 44 animals were shipped from the CDC site (Dr. O’Callaghan) to UofT (Dr. McGowan) on June 19th 2016. This group was composed of 8 saline treated animals (T0 control), 8 CORT treated animals (T1 condition), 9 DFP treated animals (T2.1 condition) and 10 DFP+CORT treated animals (T2.2 condition). Mice were treated with CORT (200 mg/L 0.6% EtOH) for 4 days in the drinking water. At day 5, mice were treated with DFP (3.0 mg/kg s.c.) and sacrifice 6 hours later.

Table 1. Animal dosing schedule:

Groups	4 Day Treatment	Time Points	
		Day 5 (sac -6 hours post dosing)	21 Day (sac – 6 hours post dosing)
1	Water	Saline (N=10) [8 received]	-
2	CORT	Saline (N=10) [8 received]	-
3	Water	DFP (N=10) [9 received]	Saline (N=10)
4	CORT	DFP (N=10) [10 received]	Saline (N=10)

5	Water	DFP -	LPS (N=10)
6	CORT	Saline -	LPS (N=10)
7	CORT	DFP -	LPS (N=10)
8	Water	Saline -	LPS (N=10)
		Total	100

Task 2: Extraction of tissue samples and blood sample processing.

Subtask 1: Shipment of blood to UofT. PBMCs from blood and hippocampus and prefrontal cortex from brain were extracted and shipped from CDC to U of T, as described above.

Subtask 2: Extraction of blood and brain samples. PBMC were extracted at CDC, however very few PBMCs were obtained. As a result, RNA and DNA yield and quality from PBMCs were inconsistent between samples during this reporting period.

Subtask 3: Blood sample tissue preparation. Due to the difficulty in obtaining sufficient RNA/DNA yield from extracted PBMCs, we have since concluded that whole blood should be used to generate DNA libraries from blood and have begun processing these samples, that were already received, at U of T.

Subtasks 4-7: Blood samples DNA sequencing preparation/DNA sequencing/manuscript. Not yet completed.

Task 3: Extraction of tissue samples and brain sample processing.

Subtask 1: Shipment of brain to UofT. Completed.

Subtask 2: DNA extraction of hippocampus and prefrontal cortex. Completed.

We succeeded in harvesting the brain tissues (hippocampus and prefrontal cortex) described above (see Animal dosing) and have extracted good quality RNA and DNA from each of the samples above.

Subtask 3-7: DNA sequencing/Analysis/Manuscript:

We have made progress in DNA methylation and histone modification procedures in the following areas:

Bisulfite-pyrosequencing for validation of DNA methylation modifications at specific loci:
Primers have previously been designed for bisulfite-pyrosequencing to validate DNA

methylation changes in genes that are known to have altered gene expression levels in the mouse model of gulf war syndrome (O'Callaghan et al., 2015). The genes are TNF- α , CCL, IL1- β , LIF, IL6 and OSM. Primers were designed against promoter and proximal regulatory regions defined by acetylation of histone 3 lysine 27 (H3K27ac) which contained MspI restriction site (CCGG) and putative GR binding sites so that data obtained could be compared to RRBS data, which also uses MspI digested DNA for library preparation. Bisulfite-pyrosequencing has been carried out for 7-16 samples for each condition in hippocampus and prefrontal cortex. In the hippocampus no site showed significant differences between saline (T0) and the combined CORT+DFP treatment (T2.2), although there was a significant difference in mean TNF promoter methylation (ANOVA, $F(3,40) = 2.936$, $p = 0.045$) and this is due to an increase in methylation in the CORT treatment. However, this difference was very small (~1.3% difference in methylation).

In the prefrontal cortex there were three sites with significantly different methylation as assessed by ANOVA. TNF promoter site 2 ($F(3,35) = 4.441$, $p = 0.01$) shows a significant difference between CORT+DFP (T2.2) and all three of the other tested treatments (T0, T1, T2.1), with a 7.5% increase in methylation. LIF promoter site 2 ($F(3,39) = 2.948$, $p = 0.045$) showed a difference in methylation between saline (T0) and CORT+DFP (T2.2). Finally, LIF promoter site 1 ($F(3,37) = 3.269$, $p = 0.32$), shows differential methylation, driven by a difference between CORT (T1) and DFP (T2.1) treatments. This demonstrates differences in DNA methylation with the exposures used in the animal model. Importantly, each of these genes was recently found by our group to show corresponding differences in gene expression (O'Callaghan et al. J. Neurochemistry, 2015) indicating a possible mechanistic role for the changes in DNA methylation.

Reduced Representation Bisulfite Sequencing (RRBS) for genome-wide DNA methylation modification analysis: Samples have been sequenced for the frontal cortex and hippocampus, however, final analysis is still ongoing. Results to date allow for a comparison between saline (T0) vs CORT (T1) in the frontal cortex, and between saline (T0) and CORT+DFP (T2.2) in the hippocampus. The prefrontal cortex samples consist of four saline controls vs three CORT treated animals, and shows 12 differentially methylated genes, 3 of which are protein coding. One of these genes is IL12rb1, which also shows differential gene expression in our RNA-seq data, and is part of the interleukin 12 receptor complex. The hippocampus samples consisted of four saline controls vs four CORT+DFP treated animals, and showed 69 differentially methylated genes. An enrichment analysis shows that these 69 genes are enriched for genes within the Jak-STAT signaling pathway, a pathway which is usually activated by cytokines. The discovery of these genes provides evidence of novel genes within the immune pathway affected by the exposures in this animal model.

Chromatin Immunoprecipitation (ChIP) sequencing for genome-wide histone modifications: ChIP data is currently being sequenced, with results expected Nov 21 2016. In addition, this reporting period we have developed a bioinformatics analysis pipeline for the ChIP seq data to

allow rapid analysis of the sequencing results once they are obtained.

Manuscript: Not yet completed.

Task 4: Gene expression analysis on blood and brain

Subtask 1: Shipment of blood and brain samples to Nova. Prefrontal cortex samples were processed at Nova using RNA-seq.

Subtask 2: Gene expression analysis on blood and brain.

RNA sequencing: We have obtained Illumina RNA sequencing (RNA-seq) data for the prefrontal cortex in saline (T0), CORT(T1), DFP(T2.1) and CORT+DFP(T2.2) conditions. These data are important because they can be integrated with the RRBS and ChIP data to establish a chain of causality, showing how alterations in epigenetic marks cause changes in gene expression genome-wide. Our analysis of the RNA-seq data showed that 206 genes are significantly differentially expressed in the combined CORT+DFP condition, compared to either treatment alone or saline. This shows that combining the two conditions causes physiological differences that are not accountable to either acting alone. We carried out pathway analysis on these genes using the R packages clusterProfiler and DOSE (Yu et al., 2012, 2015).

For the 206 differentially expressed genes, we found that 70 are annotated in the KEGG pathways database. These 70 are enriched for 12 different KEGG annotation (BH adjusted p-value ≤ 0.05).

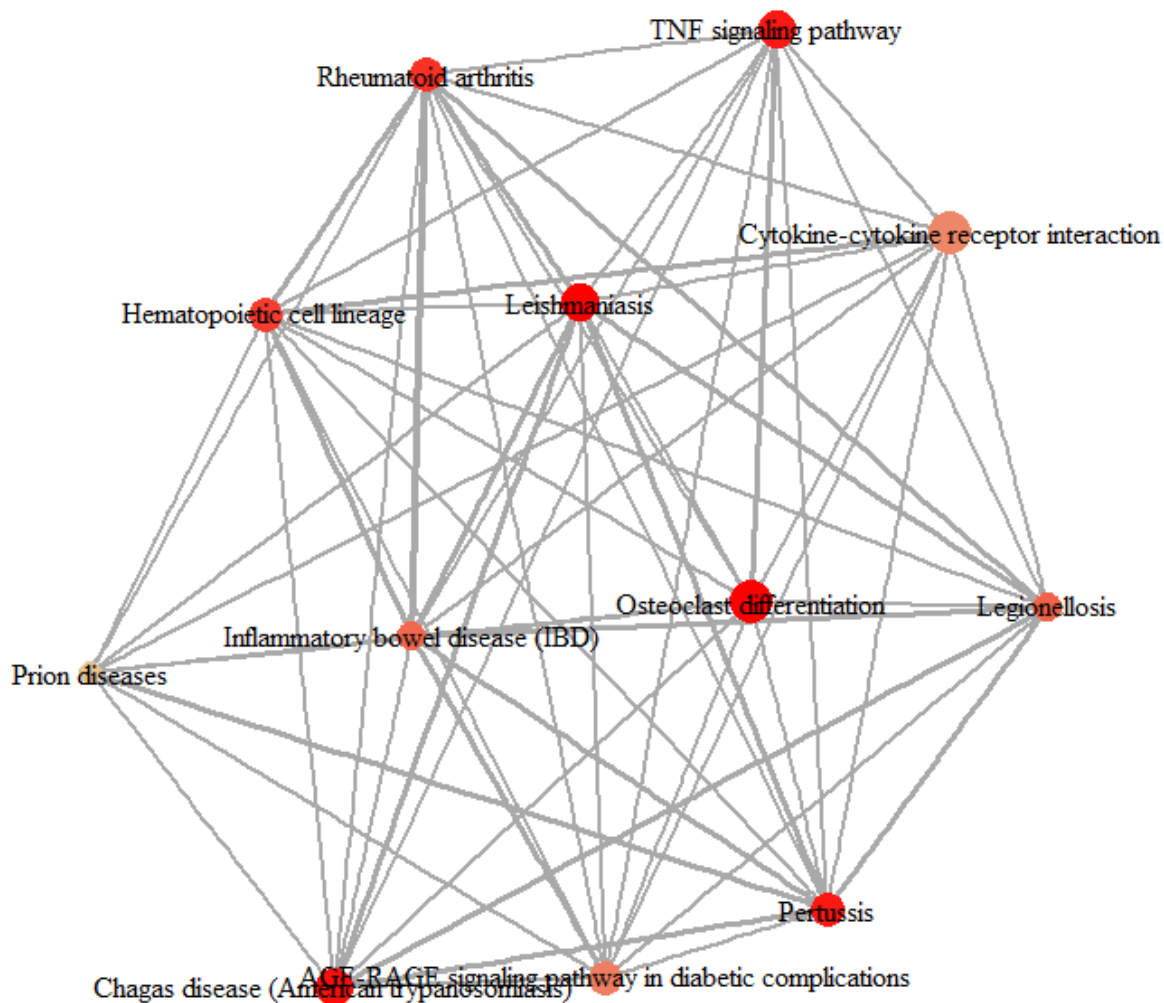


Figure 1: Kegg pathways significantly enriched BH adjusted p -value ≤ 0.05) in annotated genes differentially expressed in the CORT+DFP combined treatment.

As can be seen in Figure 1, these are mainly immune related annotations, either the cause of an immune response or the response itself. The exceptions are osteoclast differentiation and hematopoietic cell lineage. It is unclear why osteoclast differentiation appears in a brain tissue sample, and could simply be due to overlapping pathways, whereas hematopoietic cell lineage points towards microglia, which are derived from hematopoietic stem cells. These results indicate that the combined CORT+DFP treatment is increasing the immune response.

The 206 genes were also examined for gene ontology (GO) biological process (BP) annotation. This showed that 193 of our 206 genes are annotations, and 17 annotations have both a BH adjusted p and q values ≤ 0.05 .

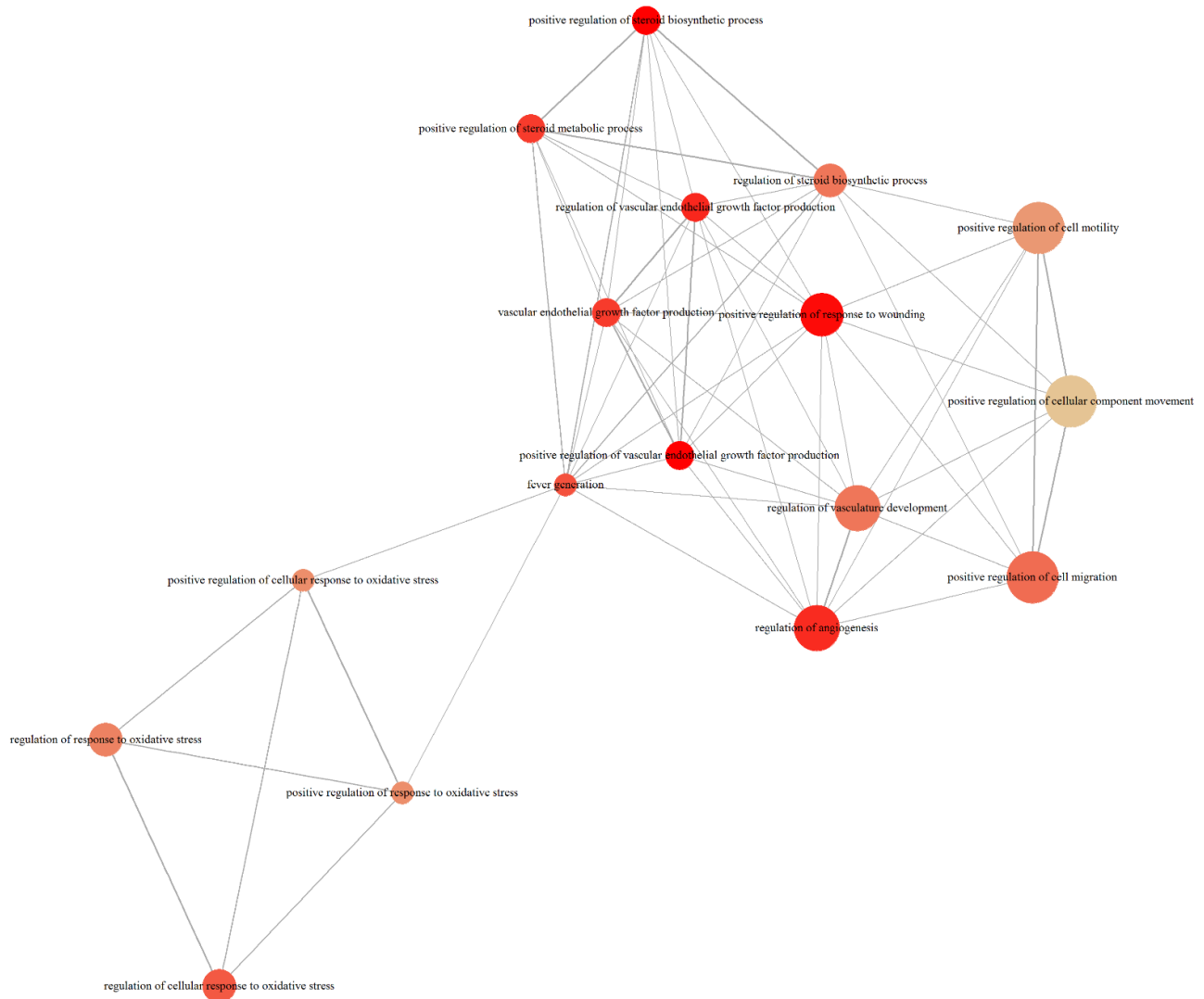


Figure 2: Gene ontology, biological process enriched in the annotated genes differentially expressed in the CORT+DFP treatment

A number of the GO annotation are clearly related to immunity such as ‘fever generation’, ‘positive regulation of response to wounding’ and ‘regulation of response to oxidative stress’. Other annotations, such as growth factor and cell mobility related annotations, may be indicative that the system is priming to repair any damage caused by the insult which triggered the immune response.

An enrichment analysis was also carried out to determine if our gene list was enriched for genes known to be specific for a subset of CNS cells. We found that our list was enriched for microglia unique genes, but not for neuron, endothelial cell, oligodendrocyte or astrocyte specific genes. Again, this points to a specific change in the neuroimmune response, as microglia are the primary immune cells of the CNS.

Fluorescence-assisted Cell Sorting (FACS) of neurons and glia from brain tissue: As mentioned in our previous report, DNA methylation is known to differ between neurons and glial cells (Iwamoto et al., 2011; Kozlenkov, NAR, 2014), the primary cell types in the brain. Work by Dr. O'Callaghan (Co-PI on this project) has indicated that glia cells may drive changes in expression associated with the exposures used in this project. This is also supported by our work in this quarter examining DNA methylation (pyrosequencing and whole-genome sequencing) and RNA-seq gene expression analysis, which shows enrichment for immune related and microglia specific genes.

This quarter, we have been optimising a FACS protocol to specifically isolate microglia, allowing us to interrogate the epigenetics of this important cell type. This work is ongoing, with some delay caused by the changeover in personnel (please see additional information below). Pat Ng, a research technician, has been recruited to facilitate this work in collaboration with the new postdoctoral fellow, Dr. David Ashbrook.

Gene expression for hippocampus and blood: not yet completed.

Task 5: Analysis of epigenetics and gene expression across tissue types

Not yet completed.

Additional Information:

Personnel changes: We have had personnel changes in PDFs (Dr. Hing to Dr. Ashbrook) and research technicians that resulted in a delay in the project due to the lack of continuity between the departure and arrival of the PDF and research technicians.

Trainee recruitment for bioinformatics: We have recruited Dr. David Ashbrook, postdoctoral fellow. Pat Ng, research technician.

Task 5 Site visits: Visit to Fort Lauderdale, FL (Co-PI site is Nova Southeastern) as part of IAME/CFS meeting, hosted by Nova, to meet with Drs. Broderick (Co-PI) Oct 27- 2016. Co-PI and PI arranged to meet with other members of the GWIRP consortium group to facilitate interaction, plan data integration experiments in Task 5, and discuss plans for data integration with data available from the larger GWIRP consortium group, of which all of the Co-PIs are members.

What opportunities for training and professional development has the project provided?

This project has provided technical training for postdoctoral fellow Dr. David Ashbrook and research technician Pat Ng.

How were the results disseminated to communities of interest?

Some of our preliminary results have been presented at the CEEHRC 'Epigenetics in development and disease conference' held Sept 18-21 in Esterel, Quebec, Canada. Poster presentation: "Epigenetic impacts of stress priming of the neuroinflammatory response to sarin surrogate in a mouse model of Gulf War Illness" authors: Ashbrook, D., Hing, B., Shao, L., O'Callaghan, J. and McGowan P.O.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period we will receive final RRBS and ChIP data for the brain for timepoints T0-T2, allowing us to examine the epigenetic effects of our treatment on the brain, and relate this to the previously collected gene expression data. Validation of gene expression data for hippocampus will be done by Nanostring at Nova. We will also receive tissue for timepoint T3 to allow us to model changes in epigenetic and gene expression over time. In addition, we will extract DNA/RNA from blood cells, prepare libraries, and perform RNA/DNA sequencing of blood, allowing us to examine gene expression and epigenetics of blood. These data will be critical to making comparisons with human data collected under the larger GWIRP consortium.

Impact

What was the impact on the development of the principal discipline(s) of the project?

The techniques we developed will serve our project as well as other DoD projects. Our data on CORT treatment will allow us to integrate epigenetic and gene expression data, providing information on the effects of stress hormones in more general settings.

What was the impact on other disciplines?

This work feeds back into our thinking of how certain gene regulatory circuits might fail in sister projects under the broader umbrella of the consortium.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

Changes/Problems

Changes in approach and reasons for change

As mentioned above, we have identified microglia as the primary cell type driving the neuroimmune changes we see. We are therefore piloting a protocol to enable cell separation by Fluorescence-assisted cell sorting (FACS) specifically for microglial cells (see Section 2 of 'Major Activities' above for results).

Actual or anticipated problems or delays and actions or plans to resolve them

In Dr. McGowan (PIs) lab, the previous Postdoctoral Fellow on the project (Dr. Ben Hing) unexpectedly left the project at short notice, and therefore a new Postdoctoral Fellow had to be recruited and trained. Further, the graduate student involved in the project (Ms. Christine Lum) graduated in Oct 2015. This delayed the progress of the project due to the lack of continuity in personnel. Dr. Ashbrook was hired in Jan 2016 as a postdoctoral fellow and Mr. Pat Ng was hired as a research technician (from June 2016) to resolve personnel shortages.

We have had unanticipated difficulty in isolating DNA and RNA of sufficient yield and quality from PBMCs for downstream analysis. We are in the process of optimising this on the current biological samples. An alternative approach is pool PBMCs from several animals under the same treatment conditions, which we will have sufficient tissue to do upon receipt of the final tissue shipment from CDC, or to use whole blood if necessary to increase DNA/RNA yield and purity.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

Products

Nothing to report.

Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Patrick McGowan, PhD
Project Role:	Principal-Investigator (UofT site PI)
Research Identifier:	none
Nearest person month worked:	3

Contribution to Project:	Lead PI overseeing the project. Direct supervision of molecular biology studies related to epigenetics data.
Funding Support:	

Name:	Gordon Broderick, PhD
Project Role:	Co-Investigator (NSU site PI)
Research Identifier:	eCommons: gbroderick
Nearest person month worked:	0.36
Contribution to Project:	Head of computational biology. Has worked on the computational models for animal and human research to assist in protocols and findings.
Funding Support:	NIH, VA

Name:	Mariana Morris, PhD
Project Role:	Co-Investigator
Research Identifier:	eCommons: mariana
Nearest person month worked:	0.36
Contribution to Project:	Overseeing the animal protocols and in charge of the animal research. Contributes technical expertise in animal experimentation. PI of GWIRC project
Funding Support:	NIH

Name:	Benjamin Hing, PhD
Project Role:	Postdoctoral fellow
Research Identifier:	None.
Nearest person month worked:	2
Contribution to Project:	Active in epigenetic assays

Funding Support:	
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Name:	Ms. Christine Lum
Project Role:	Graduate student
Research Identifier:	None
Nearest person month worked:	1
Contribution to Project:	Active in epigenetic assays
Funding Support:	

Name:	Dr. David Ashbrook
Project Role:	Postdoctoral fellow
Research Identifier:	None
Nearest person month worked:	10
Contribution to Project:	Active in epigenetic assays and RNA assays
Funding Support:	

Name:	Mr. Pat Ng
Project Role:	Research Technician
Research Identifier:	None
Nearest person month worked:	2
Contribution to Project:	Active in epigenetic assays
Funding Support:	

Name:	Mr. Patrick Gourdet
Project Role:	Research Programmer

Research Identifier:	None
Nearest person month worked:	10
Contribution to Project:	Active in data analysis and software platform design
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Name:	Centers for Disease Control and Prevention National Institute for Occupational Safety and Health
Location:	1095 Willowdale Road Morgantown, WV 26505
Contribution:	Chemical toxicology project collaboration
Financial:	None
In-kind Support:	None
Facilities:	None
Collaboration:	Partner's staff works with project staff in the project.
Personnel Exchanges:	None
Other:	None

Name:	Nova Southeastern University
Location:	Institute for Neuro-Immune Medicine University Park Plaza 3440 South University Drive

	Fort Lauderdale, FL 33328
Contribution:	Genomic profiling and computational analyses
Financial:	None
In-kind Support:	None
Facilities:	None
Collaboration:	Partner's staff works with project staff in the project.
Personnel Exchanges:	None
Other:	None

Other.

Nothing to report.

Special Reporting Requirements

Nothing to report.